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13. ABSTRACT *(Maximum 200 Words)*

The central objective of this proposal is to express the DNA-binding domain of poly(ADP-ribose) polymerase (PARP) under control of prostate tissue-specific promoter in prostate cancer cells and sensitize them to radiotherapy or chemotherapy. Here we describe the strategy for cloning the 5'-regulatory elements (1.3 kb enhancer and 0.6 kb promoter) of the human gene for prostate specific antigen (PSA). Further, we developed the recombinant plasmids that contain cDNA encoding for DNA-binding domain of PARP (PARP-DBD) downstream of the human cytomegalovirus (CMV) promoter, pCMV-DBD/F, or PSA promoter/enhancer, pPSA(EP)-DBD/F. These plasmids allow expression of the PARP-DBD protein in prostate carcinoma cells both, in constitutive and in androgen-inducible fashion. The pCMV-DBD/F construct was assayed for its ability to direct synthesis of appropriately sized FLAG-fusion protein in LNCaP prostate carcinoma cells. The availability of tissue-specific expression vectors expressing pro-apoptotic protein (PARP-DBD) offers a feasible approach for prostate cancer gene therapy.

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INTRODUCTION

Radiation therapy is an important treatment modality of prostate cancer, a second leading cause of death among men in the United States. However, its effectiveness is limited due to intrinsic resistance of tumor cells to ionizing radiation. This study will focus on the unique properties of the DNA-binding domain (DBD) of poly(ADP-ribose) polymerase (PARP) as a potent molecular radiosensitizer. We and others have previously demonstrated that genetically engeneered PARP-DBD is critically involved in DNA damage repair by acting as a *trans*-dominant inhibitor of PARP activity and that its overexpression in mammalian cells sensitizes them to DNA-damaging drugs and ionizing radiation. The central objective of the proposal is to express the DNA-binding domain of PARP under control of prostate tissue-specific promoter in prostate cancer cells and sensitize them to radiotherapy or chemotherapy. **We hypothesize that the sustained presence of the PARP-DBD in prostate tumor tissue will kill cells via apoptosis in response to massive DNA damage induced by ionizing radiation or genotoxic drugs.**

To test this hypothesis we will utilize the prostate-specific antigen (PSA) promoter to direct the PARP-DBD expression to prostate cancer cells. The regulatory region of the PSA gene has been demonstrated to show features that are fundamental to the development of expression vectors for prostate-specific gene therapy: tissue specificity and androgen responsiveness. Using PSA-producing cells (LNCaP) and cells that do not express PSA (PC-3) as the primary experimental model system we propose the experimental approach designed to: 1) produce prostate carcinoma cell sublines which allow androgen-inducible, high-level expression of the PARP-DBD and 2) test the DNA-binding domain of PARP as a molecular sensitizer for improving responses of prostate tumor cells to gamma radiation and DNA-damaging drugs. The completion of experiments proposed in this project will contribute to the development of complementary biotherapeutic approaches in the treatment of prostate cancers, which fail local-regional therapy.

ANNUAL REPORT

I. ORIGINAL STATEMENT OF WORK

The proposed studies are designed to explore the potential of novel combination therapy that would utilize the tissue-specific (prostate) and radiation-specific (damages in DNA) gene therapy for prostate cancer.

Task 1. To establish prostate cancer cell lines stably expressing PARP-DBD under control of PSA promoter regulatory elements (months 1-19)

i. develop a series of plasmids to drive prostate tissue-specific expression of PARP-DBD gene (months 1-8)
ii. produce PARP-DBD expressing sublines from LNCaP prostate carcinoma cell line (months 9-13)
iii. test tissue-specificity and responsiveness of PARP-DBD expression to androgens (months 14-19)

Task 2. To investigate the potential of PARP-DBD protein for sensitization of prostate cancer cells to ionizing radiation and DNA-damaging drugs (months 19-36)

i. test the PARP-DBD expression levels for efficiency to inhibit PARP activity and DNA damage repair following gamma radiation and drug treatments (months 19-24)
ii. investigate the effects of PARP-DBD expression on cell viability, cycle progression and apoptosis induction post-irradiation (months 24-31)
iii. determine whether cell sensitization by PARP-DBD depends upon the type of DNA damage inflicted on the cells (months 26-32)
iv. conduct radiation survival curve analysis on prostate cancer cell lines expressing differential levels of PARP-DBD to assess its radiosensitizing ability (months 28-36)

II. RESEARCH ACCOMPLISHED

A. Cloning of the PSA promoter region and construction of the PARP-DBD expression plasmids

The 5'-regulatory sequences of the human PSA gene have been cloned (Riegman *et al., 1*991). Deletion analysis of this region identified a minimal (core) promoter region (-320 bp to +12), strong upstream enhancer (-5824 bp to -3738) and the presence of down-regulating elements within the central region (-4136 bp to -541) (Pang *et al.,* 1995; Schuur *et al., 1996*; Pang *et al.,* 1997). The 5'-enhancer linked to minimal core promoter has been shown to confer (i) prostate tissue specificity, (ii) androgen dependence, and (iii) enhanced gene expression

(Schuur *et al., 1996*; Pang *et al., 1997*). These features suggest that 5'-enhancer/core promoter is an effective combination of PSA gene regulatory sequences to drive the PARP-DBD expression in prostate cancer cells.

A PCR-generated probe (nts 1-200 of PSA cDNA) was used to screen a human placenta genomic library. Two identical clones were isolated and genomic fragments were further analyzed. The 1.3 kb fragment that contains the upstream enhancer element of the PSA regulatory region (nt - 745 to -2080) was identified by hybridization with the same probe used earlier and subcloned into pcDNA 3.1 (-) expression vector (Invitrogen). The PSA promoter region (nt -619 to +12) was amplified by PCR using human placenta genomic DNA as a template and the 20 bp primers: 5'-GGTCTGGAGAACAAGGAGTG (forward primer) and 5'-TCTCCGGGTGCAGGTGGTAA (reverse primer). The resulting PCR product was directly cloned in pCRII vector (Invitrogen) and sequenced to verify its fidelity. The 1.1 kb EcoR I - Hind III fragment of the human PARP cDNA encoding for DBD was isolated as previously described (Rosenthal *et al.,* 1997). Briefly, PARP cDNA fragment encompassing the region that encodes two zinc fingers of the enzyme as well as the KKKSKK nuclear localization signal and the proximal (aa 200-220) helix-turn-helix motif was amplified by PCR and cloned into bacterial expression vector pQE-30. Flow chart representing the strategy for construction of PARP-DBD expression vectors is shown in Fig. 1 (**see Appendix**).

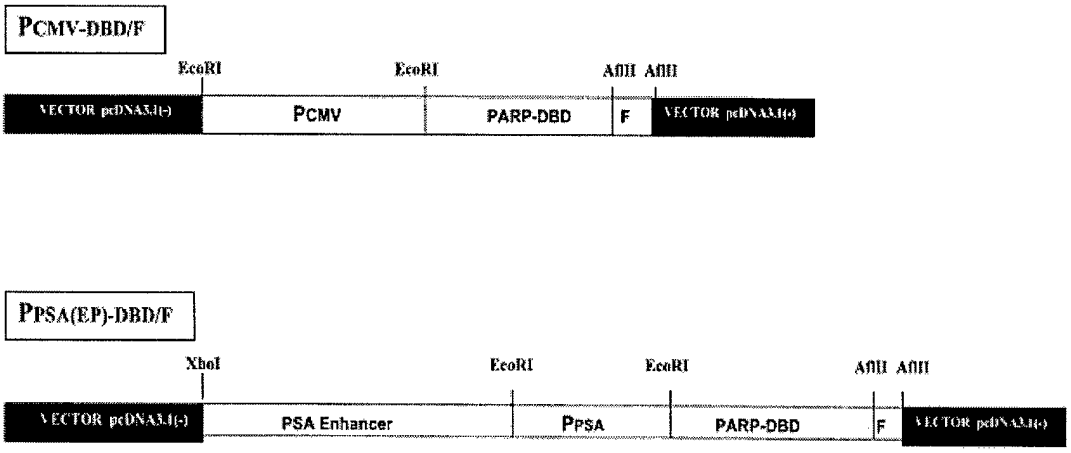

FIGURE 2. Schematic presentation of the recombinant constructs for constitutive, pCMV-DBD/F, and androgen-inducible, pPSA(EP) -DBD/F, expression of the human PARP-DBD in prostate cancer cells. PSA enhancer region, PSA core promoter (P PSA), DNA binding domain of PARP (PARP-DBD), and relevant restriction enzyme sites are indicated.

Following recombinant plasmids were constructed :

(i) The human cDNA coding for the DNA-binding domain of PARP (5'-Eco RI - Hind III) was inserted into pcDNA 3.1 (-) expression vector (Invitrogen) at EcoRI/Hind III restriction sites downstream of the human cytomegalovirus (CMV) promoter/enhancer. Subsequently, PARP-DBD was tagged at its carboxy terminus with a sequence encoding

four FLAG-epitope tags. The resulting recombinant plasmid, pCMV-DBD/F, permits constitutive expression of human PARP-DBD under control of the CMV promoter (Fig. 2).

(ii) The pCMV-DBD/F plasmid was modified to remove Nru I-Pme I fragment that contained the CMV promoter sequences giving rise to pΔCMV-DBD plasmid. This vector is used as a control in transient and stable transfections.

(iii) Next, two basic vectors for expression of the human PARP-DBD under control of the PSA gene regulatory elements were generated. An Eco RI fragment containing 662 bp sequence of PSA promoter was cloned into Eco RI site of pΔCMV-DBD giving rise to pPSA(P)-DBD/F. To generate a pPSA(EP)-DBD/F plasmid, a 1336 bp Xho I - Eco RV fragment of PSA enhancer was inserted upstream of PSA promoter into pPSA(P)-DBD/F at Xho I/Eco RV restriction sites. The resulting plasmid, pPSA(EP)-DBD/F (Fig. 2), permits the expression of the human PARP-DBD in androgen-inducible and PSA-dependent fashion. The integrity of all constructs was confirmed by sequence analysis.

Resulting recombinant plasmids were analyzed with restriction enzymes (Fig. 3), and sequences are confirmed to be in-frame (data not shown).

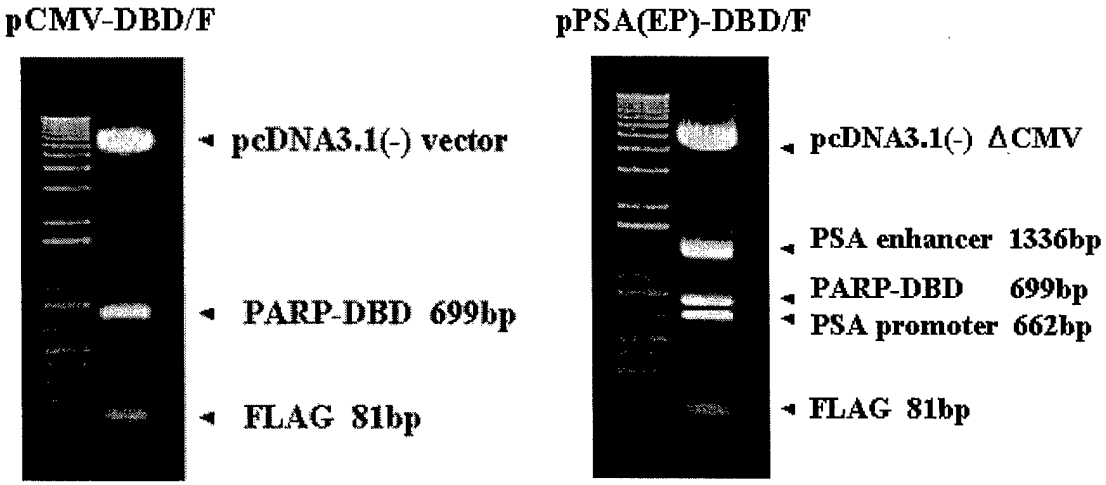

FIGURE 3. Restriction analysis of recombinant plasmids for expression of the PARP-DBD. Plasmid pCMV-DBD/F was digested with Eco RI and Afl II, and pPSA-DBD/F was analyzed by Xho I / Eco R I / Afl II endonucleases. Reaction products are separated on 0.8% agarose gel alongside the 1 kb DNA ladder (Gibco BRL).

B. PARP-DBD expression in LNCaP cells

To determine whether PARP-DBD can be expressed in prostate carcinoma, LNCaP cells transiently transfected with the pCMV-DBD/F plasmid and the FLAG-fusion proteins were detected by Western immunoblotting (Fig. 4). Human prostate cancer line LNCaP (obtained

from American Type Culture Collection, Rockville, MD) was cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37^0 C in an atmosphere of 5% CO_2 in air. DNA transfections were carried out using an activated-dendrimer reagent ("Superfect", Qiagen) essentially as we described (Soldatenkov *et* al., 1999). One day prior to transfection, $2x10^5$ cells were plated into 60 mm culture dishes. The pCMV-DBD/F plasmid (4 μg) were transiently transfected intoLNCaP cells using a ratio of DNA to transfection reagent of 1:6, for 5 hours, followed by replacing the medium containing DNA complexes with complete growth medium. 48 h after transfection cells were washed twice with cold PBS and lysed at 4^0 C for 30 min in buffer: 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 20 μg /ml aprotinin and 20 μg/ml leupeptin. Insoluble material was removed by centrifugation at 4^0 C for 30 min at 16,000 x g and protein concentrations were determined using the "Micro BCA protein assay" (Pierce). Immunoprecipitation was performed by incubating the lysate with anti-FLAG M2 monoclonal antibody agarose affinity gel (Sigma) as described (Soldatenkov *et al.*, 1997). Immunoprecipitates were washed once with the lysis buffer, twice with 0.5M LiCl-0.1 M Tris (pH 7.4), and once with 10 mM tris (pH 7.4). For immunoblotting, the immune complex was boiled in Lammeli sample buffer and subsequently resolved on SDS-4-20% gradient polyacrylamide gels (Bio-Rad), followed by Western blotting using polyclonal anti-PARP antibody (R&D System) directed against the aa 71-329 of PARP protein. The secondary antybody was donkey anti-goat IgG-conjugated to horseradish peroxidase(Santa Cruz). Signals were detected using the enhanced chemiluminescence system (Amersham).

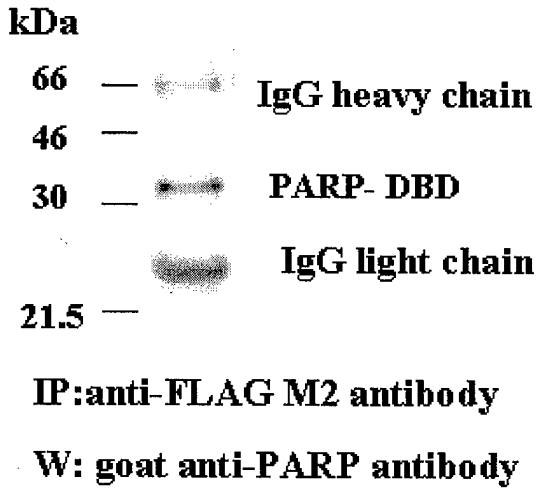

FIGURE 4. Immunodetection of PARP-DBD FLAG-fusion protein in human prostate carcinoma cells (LNCaP).

C. Establishment of stable transfected LNCaP cell lines

LNCaP cells were ransfected with PARP-DBD expressing plasmids (Fig. 2), or with control vector, pΔCMV-DBD, using "Superfect" transfection reagent (Quigen) as we described (Soldatenkov *et* al., 1999). Briefly, cells (2.0×10^5) were plated into 60 mm tissue culture dishes

coated with poly-L-lysine (Sigma) and transfected next day with 4 μg of pCMV-DBD/F or pPSA-DBD/F. DNAs cells using a ratio of DNA to "Superfect" reagent of 1:6. The transfection medium was replaced 5 h later with complete growth medium and the cells were incubated for 48 h to allow for expression of neomycin-resistance, followed by replating into selective medium containing 250 μg/ml G418 (Geneticin; GIBCO). Selection of the G418-resistant colonies is currently in progress.

KEY RESEARCH ACCOMPLISHMENTS

- 5'-regulatory elements (1.3 kb enhancer and 0.6 kb promoter) of the human PSA gene were isolated and cloned into mammalian expression vector, pcDNA 3.1(-).
- Recombinant plasmid, pCMV-DBD/F, was generated. This construct permits constitutive expression of the human PARP-DBD under control of the CMV promoter.
- Recombinant plasmid, pPSA(EP)-DBD/F, was generated. This construct permits the expression of the human PARP-DBD in androgen-inducible and PSA-dependent fashion.
- PARP-DBD expression as FLAG-fusion protein in prostate carcinoma cells, LNCaP, was demonstrated.

In addition, the work to produce PARP-DBD expressing sublines from LNCaP prostate carcinoma cell line is initiated, in accordance with the "Statement of work".

REPORTABLE OUTCOMES

PI and his consultant have reviewed the biological role for the PARP in cellular responses to DNA damage. The emphasis of this paper is on potential implications of PARP-targeted interventions for sensitizing mammalian tumor cells to radiation therapy and chemotherapy using genotoxic agents (Soldatenkov & Smulson, 2000).

CONCLUSIONS

The present study reports the construction of a prostate tissue-specific promoter and its incorporation into plasmid constructs. The availability of tissue-specific expression vector offers a feasible approach to express pro-apoptotic protein (PARP-DBD) for prostate cancer gene therapy.

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APPENDIX

1. Figure 1
2. Reprint of Journal article

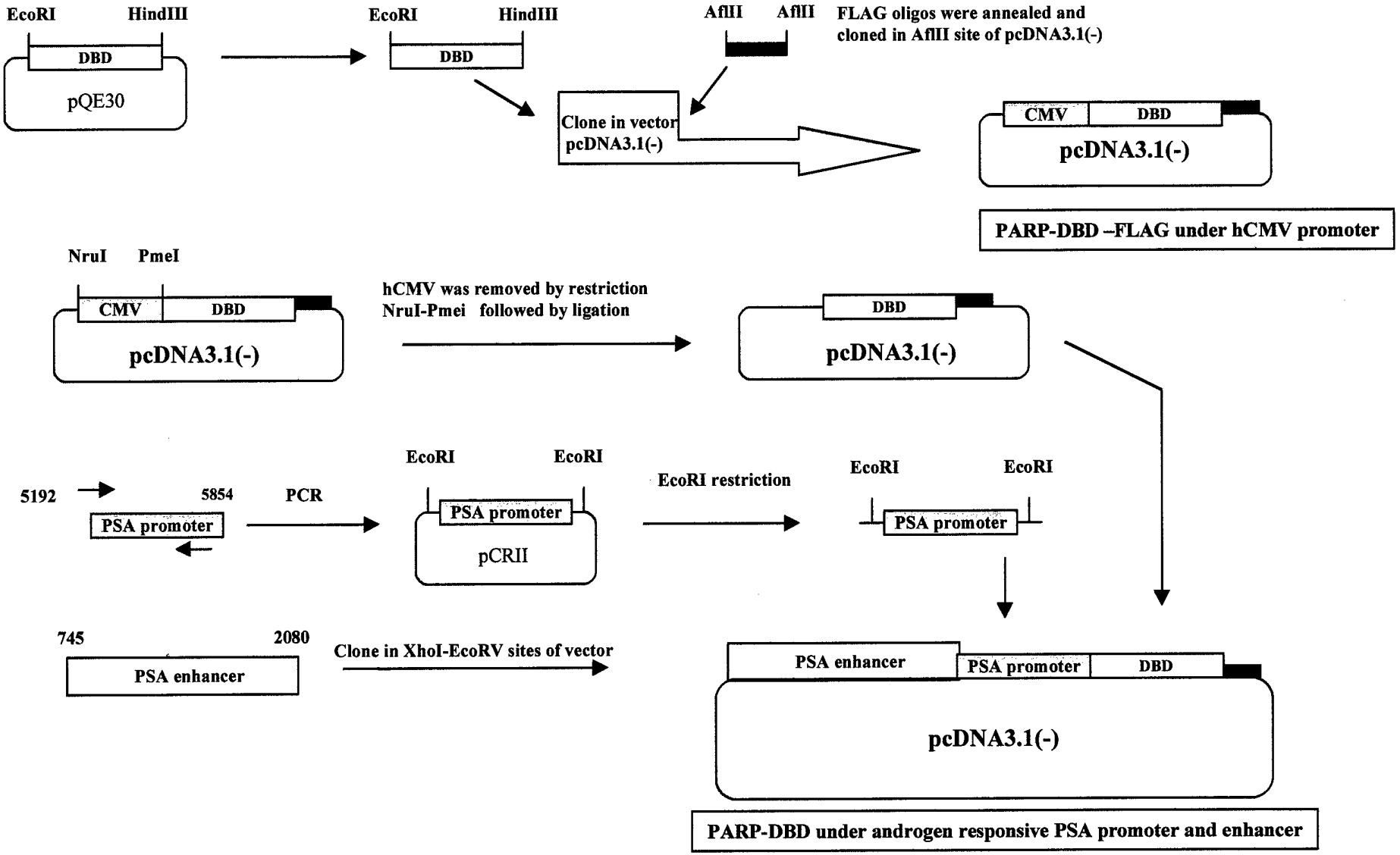

FIGURE 1. Generation of recombinant constructs for constitutive and inducible expression of the PARP-DBD. See text for explanations

Int. J. Cancer (Radiat. Oncol. Invest): **90,** 59–67 (2000)

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Poly(ADP-ribose) Polymerase in DNA Damage-Response Pathway: *Implications for Radiation Oncology*

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SUMMARY **Poly(ADP-ribose) polymerase (PARP) catalyzes the transfer of successive units of ADP-ribose moiety from NAD^+ covalently to itself and other nuclear acceptor proteins. PARP is a zinc finger-containing protein, allowing the enzyme to bind to either double- or single-strand DNA breaks without any apparent sequence preference. The catalytic activity of PARP is strictly dependent on the presence of strand breaks in DNA and is modulated by the level of automodification. Data from many studies show that PARP is involved in numerous biological functions, all of which are associated with the breaking and rejoining of DNA strands, and plays a pivotal role in DNA damage repair. Recent advances in apoptosis research identified PARP as one of the intracellular "death substrates" and demonstrated the involvement of polymerase in the execution of programmed cell death. This review summarizes the biological effects of PARP function that may have a potential for targeted sensitization of tumor cells to genotoxic agents and radiotherapy.** *Int. J. Cancer (Radiat. Oncol. Invest.) 90, 59–67 (2000).* **© 2000 Wiley-Liss, Inc.**

Key words: poly(ADP-ribose) polymerase; ionizing radiation; DNA damage repair; cell death; gene regulation

INTRODUCTION

Poly(ADP-ribose) polymerase (PARP, EC 2.4.2.30) is a chromatin-associated enzyme that catalyzes the transfer of successive units of ADP-ribose moiety from NAD^+ covalently to itself and other nuclear acceptor proteins. The catalytic activity of PARP is strictly dependent on the presence of strand breaks in DNA and is modulated by the level of automodification. On the basis of the nature and functions of acceptor proteins and the dependency of PARP on DNA strand breaks for catalytic activity, it has been suggested that PARP-dependent protein modification has a role in important cellular processes that require DNA cleavage and rejoining reactions, such as DNA replication, recombination and repair, cell cycle regulation, cell differentiation, and neoplastic transformation [reviewed in 1–5]. Much of the experimental data in support of these functions derive from studies of the effect of chemical inhibitors of polymerase activity [6–8]. Because these chemical inhibitors lack specificity and exert pleiotropic effects not directly related to PARP function, such studies remain controversial [9,10].

Recent advances in molecular biology and genetics of the PARP gene have bridged the gap be-

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tween the proposed roles for the polymerase and the factual molecular basis of its function. In addition to its role in DNA damage repair, the involvement of PARP has been implicated in regulation of gene expression [11–14] and execution of programmed cell death [15–18]. Cumulatively, these findings suggest PARP plays a fundamental role both in normal function of eukaryotic cells and in cellular response to DNA damage. This article reviews the role for PARP in cellular responses to DNA damage and attempts to integrate this knowledge with potential implications of PARP- targeted interventions for sensitizing mammalian tumor cells to radiation therapy and chemotherapy using genotoxic agents.

POLY(ADP-RIBOSYLATION) OF NUCLEAR PROTEINS

The nuclear enzyme PARP is found in almost all eukaryotic cells [1], with the only known exception being yeast [19]. PARP is a major nonhistone chromosomal protein and is present in large concentration (approximately 1 enzyme molecule per 50 nucleosomes) in eukaryotic nuclei [20]. The polymerase has a high binding affinity for blunt ends of DNA and 3′ single-base overhands compared with long overhands; the affinity of PARP for nicks in DNA is fourfold less than for blunt ends [21]. The catalytic activity of polymerase is strongly stimulated after binding of the enzyme to broken DNA ends. Benjamin and Gill [22] have shown a linear relationship between the number of nicks in DNA and polymerase activity. Moreover, the type of break is also significant for PARP stimulation [23]. Yamanaka et al. [20] estimated that only about 1% of the total polymerase molecules would be active under physiological conditions and in the absence of massive production of DNA strand breaks.

This enzyme transfers the ADP-ribosyl part of NAD^+ either to nuclear proteins or to itself to generate long, branched, and negatively charged poly(ADP-ribose) chains. When PARP is hyper(ADP-ribosyl)ated, it acquired a high negative charge, becomes repulsed from DNA, and thus is inactivated [23]. On modified proteins, poly(ADP-ribose) turns over very rapidly, with a half-life of less than 1 min [24]. The ADP-ribose polymer is hydrolyzed by poly(ADP-ribose) glycohydrolase to yield ADP-ribose, and the latter is subsequently hydrolyzed by phosphodiesterase to 5′-AMP and ribose 5-phosphate as final products [25]. Thus, the balanced actions of poly(ADP-ribose) polymerase and glycohydrolase could mediate transient physiological changes in chromatin structure and regulate functional activity of nuclear proteins.

The gene for PARP was cloned [26] and mapped to chromosome 1 at q41-q42 [27]. The cDNA encoding the human enzyme (approximately 3.7 kb length) contains an open reading frame coding for a 1,014 amino acids polypeptide with a calculated molecular weight of 113 kDa [26,27]. Three distinct functional domains are recognized by limited proteolysis of the purified enzyme: 1) a 46 kDa N-terminal domain, 2) a 22 kDa centrally located automodification domain, and 3) a 54 kDa carboxy-terminal catalytic domain [28]. The amino-terminal DNA-binding domain contains two putative zinc-binding motifs that may be responsible for the protein's specificity to bind double and single-strand breaks on DNA [29]. The automodification domain of PARP contains protein–protein binding motifs involved in recognition and stabilization of homodimeric and heterodimeric PARP-DNA complexes [30] and 15 highly conserved Glu residues that may act as automodification sites [31]. The C-terminal region is the NAD^+-binding site [32].

The binding of PARP to the broken DNA ends triggers a 500-fold stimulation of ADP-ribose polymer synthesis [33] and subsequent modification of various nuclear acceptor proteins with very strong polyanion. Poly(ADP-ribosyl)ation of proteins has profound effects on chromosomal architecture and function of chromosome-associated proteins because most of the molecular targets for PARP are DNA-binding proteins. The data summarized in Table 1 [11,12,34–53] indicate that the protein–protein or protein–DNA interactions involving PARP may have biological consequences for 1) metabolism of nucleic acids, 2) modulation of chromatin structure, 3) regulation of gene expression, and 4) maintenance of genome stability.

TRANSCRIPTIONAL REGULATION OF PARP GENE EXPRESSION

The functional involvement of poly(ADP-ribose) in various physiological phenomena such as cell differentiation, cell proliferation, and transformation of eukaryotic cells suggests that the PARP gene is highly regulated at the level of transcription. Indeed, the changes in polymerase expression levels have been demonstrated under various cellular conditions. For instance, Yamanaka et al. [20] estimated that there are 5×10^5 polymerase molecules per cell in resting peripheral blood lymphocytes; this figure increases fourfold after stimulation to proliferation with phytohemagglutinin. Furthermore, changes in levels of PARP mRNA have been shown during cell differentiation [54], cell cycle

Table 1. Protein Substrates for Poly(ADP-ribose) Polymerase

Function	Protein-acceptor	Reference
DNA metabolism	DNA polymerase α	[34]
	DNA polymerase β	[34]
	DNA ligase I	[34]
	DNA ligase II	[34]
	Topoisomerase I	[35]
	Topoisomerase II	[36]
	Ca^{2+}, Mg^{2+}-endonuclease	[37]
	Terminal transferase	[34]
	Poly(ADP-ribose) polymerase	[38]
RNA metabolism	RNA polymerase I	[39]
	RNA polymerase II	[40]
	Ribonuclease	[41]
Protein metabolism	20S Proteasome	[42]
Chromatin structure	Histones	[43]
	HMG proteins	[44]
	LMG protein	[45]
	Lamins	[46]
Gene regulation	Fos	[47]
	p53	[48]
	$TF_{II}C$	[49]
	$TF_{II}F$	[11]
	TEF-1	[12]
Other regulatory proteins	DNA-dependent protein kinase	[50]
	Numatrin/B23	[51]
	Nucleolin/C23	[52]
	PCNA	[53]

progression [55,56], lymphocyte activation [20,57], and liver regeneration [58]. However, despite numerous studies on the function of PARP in mammalian cells and recent advances in the molecular genetics of the PARP encoding gene, very little is known about mechanisms for regulation of PARP gene transcription.

The 5′-regulatory region of the PARP gene has been isolated from normal human liver and lymphoid cells [59–61] and from Ewing's sarcoma cells that express PARP at unusually high levels [62]. This upstream gene promoter exhibits features typical of TATA-deficient, G+C-rich class of promoters. Genes controlled by this type of promoter include many that are highly regulated and functionally important [reviewed in 63]. Several lines of evidence have suggested that PARP gene expression is also regulated at the level of transcription. First, previously recognized features of the PARP promoter have indicated a number of possible trans-acting factors including the presence of dyad symmetry units, SP1, and AP-2 transcription factor binding sites [59,60,64]. Next, the induction of PARP gene expression in response to cAMP and phorbol esters has been demonstrated in vitro and in vivo [60]. More recently, a mechanism of PARP gene autoregulation has been proposed, involving the specific interactions between PARP protein and cruciform structures located in the distal region of the PARP promoter [61].

PARP gene expression is maintained at relatively low levels in most human tissues, suggesting the existence of intrinsic mechanisms for the autoregulation of the endogenous content of PARP protein [54]. In contrast, Ewing sarcoma (EWS) cells accumulate PARP mRNA, protein, and polymerase activity [65] at levels that would cause the death of other cell types. Therefore, EWS cells represent a unique model for investigating PARP transcriptional regulation with regard to the identification of the transcription effectors responsible for the unusually high levels of PARP in these primitive neuroectodermal tumor cells. The 5′-flanking region of the PARP gene from EWS cells has been recently cloned and analyzed [62]. Nucleotide sequence analysis of the cloned fragment revealed no remarkable differences in the sequences reported for PARP promoter regions isolated from normal human cells [59,60]. These data suggest the enhanced levels of PARP in EWS cells relative to normal cells could be due to transcriptional upregulation of the PARP promoter rather than to sequence differences within the PARP 5′-regulatory region. Indeed, it was demonstrated that transcriptional activity of the PARP promoter correlates with protein expression levels in vitro [62,64]. One remarkable feature of the PARP gene promoter is that it contains multiple ETS-binding sties surrounding the transcription start site. The ETS multigene family encodes a class of eukaryotic transcription factors that share a highly conserved DNA-binding sequence, referred to as the ETS domain [reviewed in 66]. Recently, it has been demonstrated that ETS1 transcription factor is capable of transactivating the PARP promoter in vitro and that PARP gene expression can be modulated in cells stably transfected with antisense Ets1 cDNA [62].

Although these data suggest the existence of a variety of regulatory factors for PARP gene expression, no other endogenous PARP transactivators have been identified to date. Additional studies are required to understand the role of transcriptional factors and cis-acting elements in the regulation of the PARP gene expression. These investigations may provide an approach for the manipulation of endogenous PARP levels in human tumor cells and, therefore, for the modulation of their response to ionizing radiation and DNA-damaging drugs to improve the outcome of antitumor therapies.

PARP SIGNALING DOWNSTREAM OF DNA BREAKS

Initial evidence supporting functional involvement of PARP in DNA repair and maintenance of genomic stability has been obtained from studies using PARP competitive inhibitors (i.e., benzamide and its derivatives). Treatment of cells with chemical PARP inhibitors slows DNA repair, increases the activity of sister chromatid exchanges, and considerably increases the cytotoxicity of DNA-damaging treatments [2,4,8,67]. Although these data indicate that PARP may play a pivotal role in DNA damage repair, the limited specificity of PARP chemical inhibitors often raises questions about the validity of the results and interpretation of these studies [9,10]. Cloning the PARP gene [26,27] has allowed circumvention of most of these problems by using genetically engineered models both in vivo and in vitro. Some of these molecular approaches include the depletion of endogenous PARP protein by antisense RNA induction, the use of deletion mutants of PARP, the use of "knockout" mice with disrupted PARP gene, trans-dominant inhibition of PARP activity by over expression of its DNA-binding domain, and expression of the caspase-resistant PARP mutant in mammalian cells [reviewed in 68, 69–72].

Cell culture systems have demonstrated that PARP is involved in numerous biological functions, all of which are associated with breaking and rejoining DNA strands [68]. Eukaryotic cells expressing PARP antisense cDNA have a pronounced lag in initiation of DNA repair, which results in altered chromatin structure and reduced survival after exposure to DNA-damaging agents [73]. It has been hypothesized that PARP cycles between an unmodified form, which blocks DNA strand ends, and a modified form, which is released from DNA, thereby allowing access of repair enzymes [4]. The "PARP cycling" was recently demonstrated in an in vitro DNA repair system using deletion mutants of PARP [74].

Mice lacking PARP as a result of gene disruption exhibit diverse phenotypes. Whereas animals of one strain show epidermal hypertrophy and obesity [75], those of another strain exhibit growth retardation, aberrant apoptosis, and increased sensitivity to DNA-damaging agents [76]. Furthermore, immortalized fibroblasts derived from exon 2 PARP knockout mice (PARP–/–) exhibit mixed ploidy, including a tetraploid cell population, indicative of genomic instability [77]. Comparative genomic hybridization revealed gains in regions of chromosomes 4, 5, and 14, as well as deletion of a region of chromosome 14 (encompassing the Rb tumor-suppressor gene) in both liver tissue and immortalized fibroblasts derived from the PARP–/– animals. Neither the chromosomal gains nor the tetraploid population were apparent in PARP–/– cells that had been stably transfected with PARP cDNA [77], implicating PARP in the maintenance of genomic stability.

The possible involvement of PARP in cell-cycle checkpoint mechanisms after DNA-damaging treatments has long been suggested [55,56,78]. Excessive turnover of poly(ADP-ribose) in response to DNA damage depletes cells of their NAD^+ and at the same time or shortly thereafter, ATP levels drop [67]. This depletion leads to an overall decrease of cell metabolism and slows down the rate of cell proliferation, thereby strengthening the efficiency of DNA damage repair [79]. However, this effect is not simply the result of a generalized decrease in intracellular ATP levels, but likely to be caused by impaired function of cell-cycle regulatory proteins. Recently, Masutani et al. [80] demonstrated in vitro that PARP can directly block the cell cycle under DNA-damaging conditions by inhibition of cdk activity on pRB-phosphorylation. Furthermore, a functional association of PARP and tumor-suppressor protein p53 has recently been demonstrated. It was shown that p53 is poly(ADP-ribosyl)ated in vitro by purified PARP [81], and that PARP is required for rapid accumulation of p53, activation of p53 sequence-specific DNA binding, and its transcriptional activity after DNA damage [82]. In turn, the accumulation of p53 leads to inhibition of cell-cycle progression, thereby preventing the proliferation of damaged cells [83].

Taken together, these data suggest that PARP is an important element of cellular response to genotoxic stress acting as a component of the DNA-repair machinery and as part of the checkpoint pathway, thereby preventing cells carrying damaged DNA from unrestrained DNA replication or entering mitosis (Fig. 1). Therefore, inactivation of PARP may have therapeutic implications, because it will render cell particularly sensitive to DNA damaging agents due to impairment of cellular recovery from DNA damage.

PARP AND PROGRAMMED CELL DEATH

The "cytoprotective" function of PARP is dramatically changed when the massive DNA damages cannot be effectively repaired. Damaged cells that fail to pass the DNA damage checkpoint are elimi-

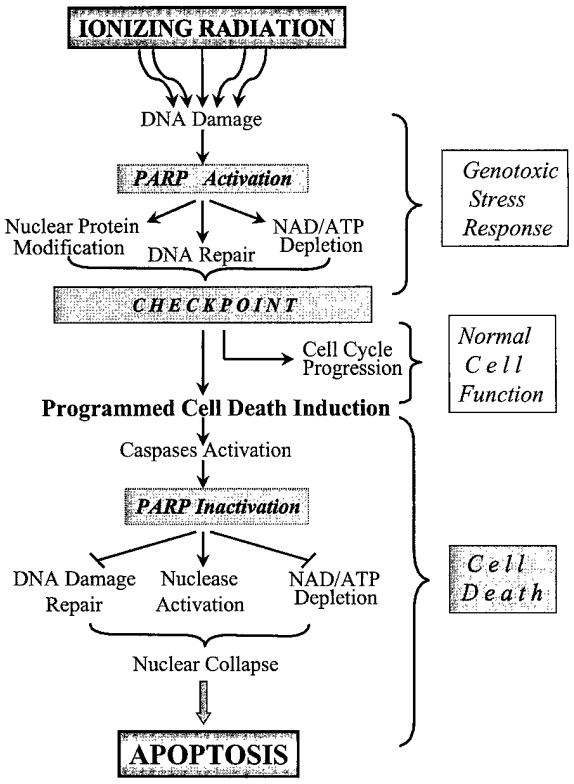

Fig. 1. Poly(ADP-ribosyl)ation of nuclear proteins in cellular response to DNA damage.

nated by a programmed self-destruction process commonly termed apoptosis [84]. Upon activation of cellular suicide (apoptosis), PARP is recruited to participate in the execution of the cell death program, serving as a "death substrate."

The requirement of PARP for execution of apoptotic pathways has been recently demonstrated by using immortalized fibroblasts derived from wild-type (PARP+/+) and PARP knockout (PARP–/–) mice [85]. Whereas immortalized PARP+/+ cells showed the early burst of poly(ADP-ribosyl)ation and rapid apoptotic response to anti-Fas treatment, PARP–/– fibroblasts exhibited neither the early poly(ADP-ribosyl)ation nor any of the biochemical or morphological changes characteristic of apoptosis when similar treated. Stable transfection of PARP–/– fibroblasts with wild-type PARP rendered the cells sensitive to Fas-mediated apoptosis. These results suggest that PARP and poly(ADP-ribosyl)ation may trigger key steps in the apoptotic program.

It has been well recognized that limited proteolysis of PARP by caspases family of cysteine proteases is an early event or perhaps a prerequisite for the execution of programmed cell death in various mammalian cells [15–17,86]. The caspase-specific DEVD motif resides adjacent to the nuclear localization signal of PARP protein. Cleavage of PARP at this site results in the separation of the two zinc-finger DNA-binding motifs in the amino terminus of PARP from the automodification and catalytic domains located in the carboxyl terminus of the enzyme [17]. Consequently, this cleavage excludes the catalytic domain from being recruited to the sites of DNA fragmentation during apoptosis and presumably disables PARP from coordinating subsequent repair of genome maintenance events [74]. Recently, the irreversible finding of the 24 kDa proteolytic fragment of PARP to broken DNA ends has been directly demonstrated by atomic force microscopy [87]. The significance of PARP cleavage and DNA-binding domain (DBD) of PARP (PARP-DBD) accumulation for execution of apoptosis has been investigated by using stable cell lines constitutively expressing PARP-DBD [18,70]. Enforced expression of the N-terminal fragment of PARP containing the DBD in cultured mammalian cells led to trans-dominant inhibition of the resident PARP activity and delay in DNA strand break rejoining. Furthermore, exposure of PARP-DBD–expressing cells to DNA damaging agents and ionizing radiation resulted in a marked reduction of cell survival, increased frequency of sister chromatid exchanges, inhibition of cell proliferation, and induction of apoptosis [18,70].

PARP cleavage by caspase(s) occurs early in apoptosis, before or soon after the appearance of internucleosomal fragmentation of DNA [15–17], a biochemical hallmark for programmed cell death. Although several nucleases are implicated in the mechanisms of chromosomal DNA disintegration in dying cells [reviewed in 88], it has been suggested that Ca^{2+}/Mg^{2+}-dependent endonuclease (CME) is responsible for cleavage of genome DNA at internucleosomal sites [89] during the late phase of apoptosis execution in most of the eukaryotic cells. This endonuclease is maintained in a latent form by poly(ADP-ribosyl)ation [37]. Consequently, inactivation of PARP by caspases may result in CME derepression and thereby promote fragmentation of genome DNA. The plausibility of such a mechanism has been demonstrated in vitro using endonucleolysis of isolated nuclei as a model in the presence of PARP inhibitors [90]. In addition, the inactivation of poly(ADP-ribosyl)ation might facilitate the accessibility of endonucleases to chromatin in dying cells. Indeed, downregulation of PARP expression by antisense mRNA delivery to cells resulted in an increased accessibility of micrococcal nuclease to nuclear DNA in chromatin [73].

Recent studies suggest that apoptosis is an energy-requiring process and that an intracellular adenosine triphosphate level influences the mode of cell death—apoptosis or necrosis [91]. Rendering PARP catalytically inactive by caspase cleavage would prevent the decrease in the content of NAD^+ and ATP, thus providing the source of intracellular energy needed for execution of the cell death program. This idea has been supported in recent studies designed to prevent PARP proteolysis by introduction of point mutations into the DEVD cleavage site to produce the "uncleavable" mutant protein. The mammalian cells expressing the caspase-resistant PARP protein in a PARP-null background exhibited accelerated tumor necrosis factor-alpha–induced cell death and increased apoptosis [92,93]. These data suggest that PARP cleavage prevents necrosis associated with depletion of NAD^+ and ATP to ensure appropriate execution of programmed cell death. However, the PARP-mediated changes in intracellular NAD^+ and ATP content do not always occur in cells undergoing apoptosis [94,95]. Therefore, the cause-effect relationship of NAD^+ depletion to apoptosis execution should be viewed critically.

CONCLUDING REMARKS

Recent developments in molecular genetics of the PARP gene and availability of PARP-deficient cells from transgenic knockout mice allowed reevaluation of the biological functions of this unique modification of nuclear proteins in the maintenance of cell surveillance. An early transient burst of poly(ADP-ribosyl)ation in response to DNA damage and subsequent inactivation of PARP during an execution stage of apoptosis indicate that PARP has active and complex roles in mechanisms of cellular stress response and in pathways leading to programmed cell death. PARP activity appears to be necessary for maintenance of genome stability in normal living cells and during the adaptive phase of cellular response to the genotoxic stress. This "pro-life" function of PARP is switched to a "pro-death" function, when cells are not capable of enduring the sustained DNA damage in the genome and are to be eliminated via apoptosis. The cleavage of PARP that occurs during the execution phase of apoptosis might help avoid unnecessary DNA repair in dying cells, facilitate nuclear disintegration, and preserve the energy needed for the biochemical cascade of events culminating in apoptosis, thus ensuring the completion and irreversibility of the cell death process (Fig. 1). Therefore, the development of gene-engineered approaches to target-specific inactivation of PARP in mammalian cells may lower the apoptosis threshold in cancer cells, thereby enhancing the effectiveness of both chemotherapy agents and radiotherapy. This may lay the groundwork for the long-awaited translation of scientific gains from investigations on PARP function to in vivo treatment of cancer.

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